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Ultrastructures of *Colletotrichum orbiculare* in the Leaves of Cucumber Plants Expressing Induced Systemic Resistance Mediated by *Glomus intraradices* BEG110

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The colonization of an arbuscular mycorrhizal fungus *Glomus intraradices* BEG110 in the soil caused a decrease in disease severity in cucumber plants after fungal inoculation with *Colletotrichum orbiculare*. In order to illustrate the resistance mechanism mediated by *G intraradices* BEG110, infection patterns caused by *C. orbiculare* in the leaves of cucumber plants and the host cellular responses were characterized. These properties were characterized using transmission electron microscopy on the leaves of cucumber plants grown in soil colonized with *G intraradices* BEG110. In the untreated plants, inter- and intra-cellular fungal hyphae were observed throughout the leaf tissues during both the biotrophic and necrotrophic phases of infection. The cytoplasm of fungal hyphae appeared intact during the biotrophic phase, suggesting no defense response against the fungus. However, several typical resistance responses were observed in the plants when treated with *G intraradices* BEG110 including the formation of sheaths around the intracellular hyphae or a thickening of host cell walls. These observations suggest that the resistance mediated by *G intraradices* BEG110 most often occurs in the symplast of the host cells rather than in the apoplast. In addition, this resistance is similar to those mediated by biotic inducers such as plant growth promoting rhizobacteria.

KEYWORDS : Anthracnose, Induced systemic resistance (ISR), Plant growth promoting rhizobacteria (PGPR), Systemic acquired resistance (SAR), Transmission electron microscopy

Plants can generate resistance following exposure to exogenous stimuli such as a pathogen, a non-pathogen, or a chemical inducer such as DL-3-amino butyric acid (BABA) and salicylic acid (SA) (Cohen, 2002; Ryals *et al*., 1992; Sticher *et al*., 1997). This type of resistance is known as systemic acquired resistance (SAR). Some rhizobacteria can induce systemic resistance in plants and this resistance is known as induced systemic resistance (ISR) (van Loon, 2007). The rhizobacteria mediating ISR are called plant growth promoting rhizobacteria (PGPR) because they also promote plant growth after pre-inoculation in the root system (Kleopper *et al*., 1980). Both types of resistance have been fairly well characterized. For example, the signaling pathway induced by SA may play an important role in SAR (Malamy *et al*., 1990), while ethylene or jasmonic acid may have significant impacts on the signaling pathways mediating ISR (Pieterse *et al*., 2001). Also, resistance expression was illustrated in plants demonstrating SAR or ISR. In the SAR-expressing cucumber leaves, the germination rate of conidia and the rate of appressorium formation of fungal pathogen were decreased (Jeun *et al*., 2004). In contrast, defense reactions were observed in the symplast of ISR-expressing plants (Jeun *et al*., 2007).

In the last two decades, it was found that some arbuscular mycorrhizal fungi (AMF) improve plant health and also increase resistance against plant pathogens by forming endosymbiotic associations with plants (Azcón-Aguilar and Barea, 1996). For example, disease severity caused by *Phytophthora parasitica* was reduced when tomato plants were colonized by *Glomus mosseae* (Cordier *et al*., 1996, 1998; Pozo *et al*., 1996, 1999; Trotta *et al*., 1996; Vigo *et al*., 2000). Like PGPR, AMF can serve as potential biocontrol agents for crop species (Pozo *et al*., 2002). However, there have been limited studies concerning the resistance mechanisms mediated by AMF relative to those underpinning SAR or ISR.

It seems that the level of resistance may vary depending on the type of inducer. For example, disease severity of cucumber plants infected by *Colletotrichum orbiculare* was lower when plants were pretreated with BABA compared to those pre-inoculated with PGPR strains, such as *Serratia marcescens* 90-166 and *Pseudomonas fluorescens* 89B61 (Jeun *et al*., 2004). Also, in the same plants,

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BABA mediated higher resistance against the disease compared to that by *Glomus intraradices* (Lee *et al*., 2005). Treatment with BABA decreased disease severity of tomato late blight by 90%, whereas the reduction was only 50% following pre-inoculation with *Tobacco necrosis* virus (Jeun *et al*., 2000).

These differences in resistance efficacy suggest that the defense mechanism may differ depending on the inducer used for treatment. In our previous cytological study, it was revealed that the ultrastructures were different in host cells pretreated with β-amino butyric acid (BABA) and pre-inoculated with two PGPR strains in cucumber plants (Jeun *et al*., 2007). Therefore, the goal of this study was to characterize the nature of resistance against *C. orbiculare* when pre-inoculated with the mycorrhizal fungus *Glomus intraradices* BEG 110. These studies were performed by characterizing the inner tissues of cucumber plants, such as the parenchyma and spongy palisade layer, using transmission electron microscope.

Materials and Methods

Plants. Second-leaf-stage cucumber plants (*Cucumis sativus* L. cv. Eunsung) were used for all treatments. Cucumber seeds were sown in plastic pots (10 cm in diameter) filled with a commercial soil (Choroc Nala®, Bokyung Nongsang, Korea) supplemented with 10% Perlite (Parat®, Sam Son, Korea). The cucumber seedlings were grown in a greenhouse maintained at 28°C during the day and 25°C during the night.

Treatment of cucumber plants with *G. intraradices* BEG110. Preparation of *G. intraradices* BEG 110 was carried out as previously described (Lee *et al.*, 2005). *G. intraradices* BEG 110 was propagated several times using white clover grown in a substrate mix of sterilized sand and vermiculite (1 : 1) in a glass house for ten weeks. The mixture colonized with *G. intraradices* BEG 110 was added as 10% (v/v) ratio of the plastic pot containing the commercial soil. Cucumber seeds were sown in the pots colonized with *G. intraradices* BEG 110 and grown in the green house until used. The same commercial soil without *G. intraradices* BEG 110 was used as a negative control.

Pathogen inoculation and disease assessment. *C. orbiculare* was grown on green beans agar medium for 5 days until spores were formed. Ten *ml* of distilled water and a brush were used to harvest conidia. This conidial suspension was adjusted to 2.5×10^5 conidia/*ml* and supplemented with Tween 20 (100 μl per liter) to dislodge conidia from leaf surfaces. Cucumber leaves were sprayed with the conidial suspension on the second leaf-growth stage plants. The inoculated plants were kept in a humid chamber maintained at 100% RH for 24 h and then transferred to a greenhouse. The experiments were replicated on three separate occasions.

The development of lesions on the inoculated leaves was observed. Number of anthracnose lesions per leaf was counted 7 days after challenge-inoculation. Protection rate was calculated as described by (Cohen, 1994), that is the rate (%) = 100 (1 – x/y) in which x and y are number of lesions on the leaves of treated and non-treated plants, respectively. The statistical analyses of the impact of *C. orbiculare* on lesion numbers were analyzed by *t*-test using a statistic software program (Office Excel 2003, Microsoft®).

Preparation of plant tissues for transmission electron microscopy. The first leaf of 3 plants was taken 5 days after inoculation with *C. orbiculare*. The corresponding leaves of control plants were also taken. The leaves were cut into small pieces (1×3 mm^2) using a razor blade. Three sections were prepared from each leaves. Fixation, dehydration and embedding of leaf pieces were performed as previously described (Hayat, 1989). The leaf pieces were fixed in 2% (v/v) glutaraldehyde in 0.05 M phosphate buffer, pH 7.2, at 4°C for 2 h. Subsequently, the pieces were washed with the same buffer three times for 10 min each and were post-fixed in 2% (w/v) osmium tetroxide in the phosphate buffer for 2 h at room temperature. After washing with the buffer, the fixed specimens were dehydrated by treating them with a series of ethanol solutions (30, 50, 70, 90 and 100%, two treatments with each solution for 30 min each). The samples were embedded in an epoxy resin (vinylcyclohexene dioxide, ERL 4206) (Spurr, 1969) and polymerized at 70°C for 8 h. The embedded blocks were sectioned ultra-thin (60 nm) using an ultramicrotome (Reichert Ultracut, Leica). The sections were mounted on a 300 mesh copper grid.

After drying, the sections were stained with 2% (w/v) uranyl acetate in H_2O and lead citrate (Reynolds, 1963). Fungal structures in the plant tissues were examined using a transmission electron microscope (TEM; JEM-1010, JEOL, Japan) at 80 kV. The TEM images were taken using 25 mm-pixelsize digital imaging plates (IP) and delivered by an IP reader (FDL 5000, Fujifilm, Japan).

Results

Reduction in lesion numbers following treatment with resistance inducers. The number of lesions in the untreated plants was first visually observed 3 days after the fungal inoculation. The size of the lesion continuously increased until 5 days after challenge inoculation (Table 1). However, on the leaves of plants colonized with *G. intraradices* BEG110 lesion numbers were reduced, and had not developed 5 days after the inoculation (Table 1).

Table 1. Analysis of lesion numbers and protection rate on the leaves of cucumber plants colonized with mycohrriza *G. intraradices* BEG110 and untreated plants 7 days after inoculation with *C. orbiculare*

Test	Inducer[a]	Number of lesions (per leaf)	Protection (%)[c]	*t*-test[d]
Ex. 1	*G. intraradices*	31.0 ± 21.2[b]	70.5	0.0591
	Control	105.0 ± 50.4	–	
Ex. 2	*G. intraradices*	22.8 ± 8.4	64.2	0.0406
	Control	63.5 ± 30.9	–	
Ex. 3	*G. intraradices*	27.0 ± 18.0	68.1	0.0231
	Control	84.5 ± 51.5	–	
Total	*G. intraradices*	26.9 ± 15.6	68.1	0.0006
	Control	84.3 ± 44.6	–	

[a]Colonization of *G. intraradices* BEG110 is described in 'materials and methods'.
[b]Values represent means ± standard deviation of 3 separated experiments each containing 12 plants per treatment.
[c]Percentages were calculated using the formula, protection (%) = 100 (1 − x/y) in which x and y are the number of lesions on the leaves of treated and non-treated control plants, respectively.
[d]*P* values.

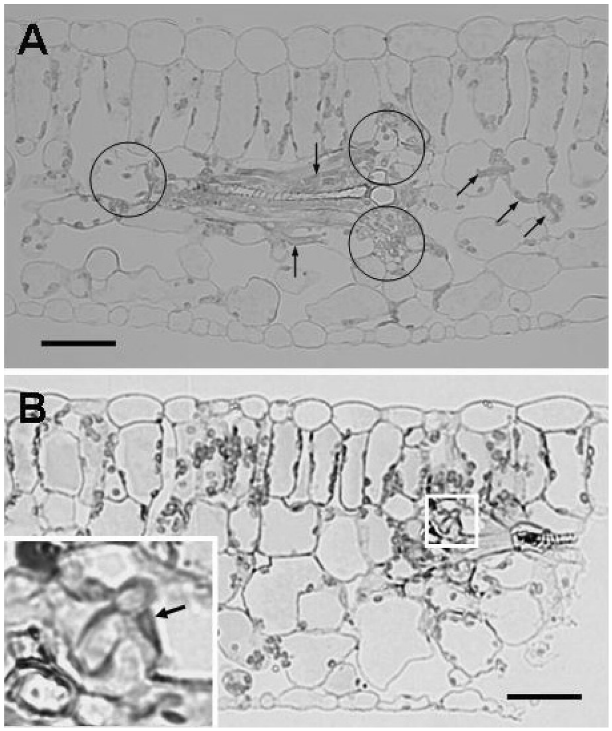

Fig. 1. Light micrographs of the infected leaves of cucumber plants 5 days after inoculation with *Colletotrichum orbiculare* (2.5×10^5 conidia/*ml*). The plants were untreated (A) or colonized with mycorrhiza *Glomus intraradices* BEG 110 (B) 5 days before the fungal inoculation. Next, the samples were semi-thin (1 μm) sectioned. Inter- or intracellular hyphae were spread throughout the leaf tissue of the untreated cucumber (A) (circles and arrows). Inter- or intracellular hyphae were rarely found in the plants colonized with mycorrhiza *G. intraradices* BEG 110 (B) (square). A collar was observed at the penetration site, indicating an active response by the host cell (arrow in the square). The length of bars indicates 100 μm.

Growth of *C. orbiculare* in the leaves of susceptible cucumber plants. Five days after inoculation, abundant inter- and intra-cellular hyphe were found in the palisade and the spongy parenchyma layer of leaves in the control plants (Fig. 1A). Also, in the vascular bundle some intercellular hyphae were observed (Fig. 2D). The infection occurred via two different phases, the biotrophic phase and the necrotrophic phase. During the biotrophic phase, the morphology of host cells was unchanged around the penetration hyphae (Fig. 2A), suggesting the absence of direct response against the pathogen. Although thickened cytoplasm in penetrated cells was sometimes observed, such a defense reaction was not enough to prevent the attack by the pathogen (Fig. 2B and 2C).

In the necrotrophic phase, the collapse of tonoplast and destruction of chloroplasts were observed (Fig. 2E and 2F). Also, unlike in the biotrophic phase, collapsed hyphae were observed in the necrotrophic phase (Fig. 2E and 2F). Most observations showed typical compatible host-pathogen interactions.

Infection structures of *C. orbiculare* in the leaves of plants whose roots were colonized by *G. intraradices* BEG110. Fungal infection structures in the leaves of plants colonized by *G. intraradices* BEG110 were different when compared to those in untreated plants. Hyphae were rarely found in the parenchyma or the spongy palisade layer of plants colonized by *G. intraradices* BEG110 5 days after fungal inoculation (Fig. 1B). Furthermore, at the penetration sites the plant cells actively reacted to the pathogen as evidenced by a thickening of cytoplasm in many cells. These active responses were observed in both the biothrophic and necrothrophic phases. Sheath was often observed around intracellular hyphae (Fig. 1B and 3A~3D). In some cases, a thickening of the cell wall around intracellular hyphae (Fig. 3E) or matrix between the cell wall and the cytoplasm of the plant cells adjusted to fungal hyphae (Fig. 3F) was observed. All of the host cytoplasm around the fungal hyphae showed evidence of a defense reaction indicating that the resistance may be expressed in the symplast of the host cells.

Discussion

In most cases, the levels of resistance triggered by chemical inducers were higher than those triggered by biotic inducers such as PGPR or mycorrhizal fungi in cucumber or potato plants (Jeun *et al.*, 2004; Kim and Jeun, 2007; Lee *et al.*, 2005; Potlakayala *et al.*, 2007). On the leaf

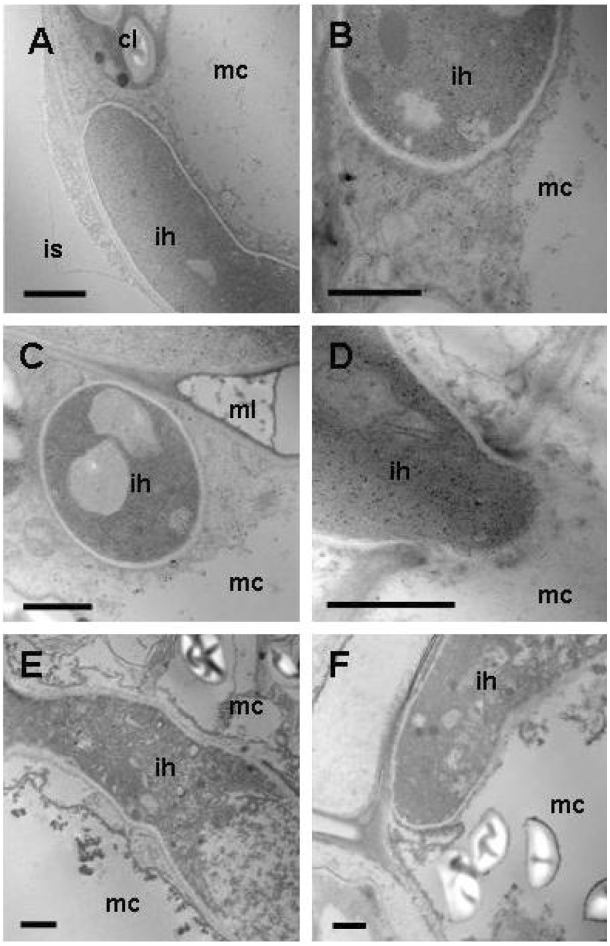

Fig. 2. Transmission electron micrographs of the leaves of untreated control cucumber plants 5 days after inoculation with *Colletotrichum orbiculare* (2.5×10^5 conidia/*ml*). (A) The intracellular hypha penetrates into a neighbor palisade host cell in the biotrophic phase (A). The intracellular hyphae were intact in the mesophyll of host cells (B and C). Intracellular hyphae were spread throughout the bundle sheath cells (D). The intracellular hyphae were denatured in the host cells in the necrotrophic phase (E and F). All bars = 1 μm. Abbreviations: ch, chloroplast; ih, intracellular hypha; mc, mesophyll cell; ml, middle lamella.

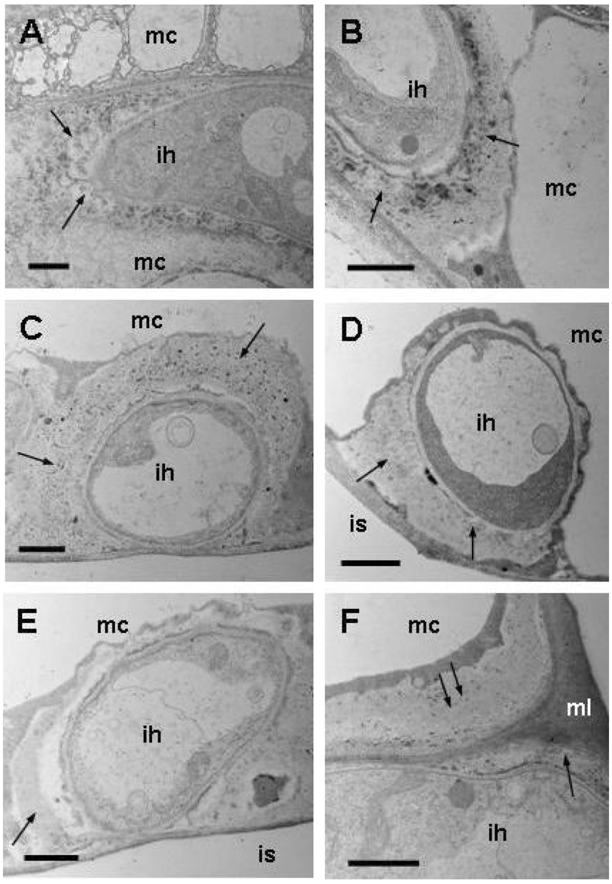

Fig. 3. Transmission electron micrographs of the leaves of cucumber plants pre-inoculated with mycorrhiza *Glomus intraradices* BEG 110 5 days after challenge inoculation with *Colletotrichum orbiculare*. Some electron non-transmissible materials accumulated around the intracellular hyphae in the biotrophic phase (A and B) (arrow). A thickening of the cytoplasm was observed around an intracellular hypha (C) (arrow). Numerous vesicles accumulated around the intracellular hyphae in the cytoplasm (D) (arrow). A barrier-like cell well was found in the necrotrophic phase (E) (arrow). The host cell wall was thickened adjacent to the intercellular hypha (F) (double arrows). All bars = 1 μm. Abbreviations: h, intercellular hypha; ih, intracellular hypha; is, intercellular space; me, mesophyll cell; ml, meddle lamella.

surfaces of cucumber plants pre-inoculated with PGPR strains *Serratia marcescens* (90-166) and *Pseudomonas fluorescens* (89B61), no decrease in germination rate or suppression of appressorium formation of *C. orbiculare* was observed unlike the leaves of untreated plants. In contrast, the germination rate and fungal appressorium formation were dramatically reduced on the leaf surfaces of the plants pre-treated with BABA or aminosalicylic acid (ASA) (Jeun *et al*., 2004). Similar results were observed on potato plants in which appressorium formation of *Phytophthora infestans* decreased following the pre-treatment of leaves with BABA, *Ps. putida* (TRL2-3), *Micrococcus luteus* (TRK2-2), *Flexibacteraceae bacterium* (MRL412) (Kim and Jeun, 2007) or *G. intraradices* (Lee *et al*., 2005). These results suggest that the resistance mechanism on the surface of the leaves might be expressed differently depending on the type of inducer.

Also, it seems that the defense reactions in the inner host cells are differently expressed according to the inducers. Jeun *et al.* (2007) have reported some differences in defense reactions between plants pre-inoculated with PGPR *Bacillus pumilus* INR-7 and those with BABA. The pre-inoculation with *B. pumilus* INR-7 caused active defense reactions in the host cells after pathogen penetra-

tion whereas BABA did not. In this study, the infection sites in the parenchyma cells of cucumber plants colonized with mycorrhiza *G. intraradices* BEG110 were observed using transmission electron microscope after challenge inoculation with *C. orbiculare*.

The infection strategy of anthracnose fungus is characterized by two phases of nutrition up-take from the host; the initial period of infection is biotrophy, and the secondary phase is characterized by hyphae spreading necro- trophically to adjacent host tissues (Bailey *et al*., 1992). In the untreated plants, most infected host cells maintained their cell structures, and organelles were not destroyed (Fig. 2A~2F). These effects are normally observed in biotrophic interactions (Green *et al*., 1994; Mendgen and Deising, 1993; Spencer-Phillips, 1997). Observations made in this study suggest that the initial biotrophic phase caused by anthracnose pathogens may last longer in the untreated cucumber plants allowing the pathogen to take up nutrients more effectively and cause a broad spread of fungal hyphae.

Unlike untreated plants, active defense reactions were observed in the plants colonized by *G. intraradices* BEG110 (Fig. 1B and 3). There was an accumulation of fiber-looking materials (Fig. 3A and 3B) or particles (Fig. 3C and 3E) in the thickened cytoplasm of the plants pre-inoculated with mycorrhiza indicating an active defense reaction. Interestingly, these observations were similar to those seen in plants pretreated with PGPR (Jeun *et al*., 2007). Similar ultrastructural results were observed in tomato plants pre-inoculated with *Tobacco necrosis virus* after challenge inoculation with *P. infestans* (Jeun, 2000) or in cucumber plants pre-inoculated with PGPR such as *S. marcescens* (90-166) and *Ps. fluorescens* (89B61) (Jeun *et al*., 2007). Based on these observations it seems that the resistance mechanism mediated by mycorrhiza may be similar to those caused by PGPR.

On the other hand, there were no apparent cellular defense responses in the symplast of plants pre-treated with BABA (Jeun *et al*., 2007). Despite the lack of any apparent cellular defense responses in the symplast, BABA is known to be an effective defense inducer; BABA pre-inoculated plants expressed strong resistance against various plant pathogens in many plants (Cohen, 2002; Zimmerli *et al*., 2000). Therefore, it is likely that BABA causes a defense reaction in the apoplast rather than in the symplast (Jeun *et al*., 2007). In fact, there were decreases of appressorium formation and germination of fungus *C. orbiculare* on the surface of cucumber plants when pretreated with BABA (Jeun *et al*., 2004; Lee *et al*., 2005).

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